Identification of a novel antigen on the apical surface of rat alveolar epithelial type II and Clara cells

GRÁINNE M. BOYLAN,1,2 JAMES G. PRYDE,2 LELAND G. DOBBS,3 AND MARY C. McELROY1,2
1Department of Physiology, Trinity College, Dublin 2, Ireland; 2Rayne Laboratory, Respiratory Medicine, University of Edinburgh, Edinburgh EH8 9AG, United Kingdom; and 3Cardiovascular Research Institute, University of California, San Francisco, California 94118

Received 16 October 2000; accepted in final form 8 January 2001

THE ALVEOLAR AND BRONCHIOLAR REGIONS of the lung are composed of a number of morphologically distinct epithelial cell types. These cell types include type I and II cells in the alveolar region and Clara and ciliated cells in the terminal bronchiolar region. Alveolar epithelial type II and Clara cells are important for repair of the epithelium in response to injury (reviewed in Ref. 25). Alveolar epithelial type II cells proliferate and differentiate to form type I cells, whereas Clara cells proliferate and differentiate to form ciliated cells (25). However, it is also likely that other epithelial cell-repair relationships exist; for example, Clara cells may also be important for alveolar repair (9).

It has long been suspected that the degree of damage to a given lung epithelial cell type is dependent on the nature of the toxic insult. McElroy and colleagues (26, 27) have recently shown that alveolar epithelial type I cell injury can be quantified by measuring the level of RTI40 in bronchoalveolar lavage fluid. RTI40 is an integral membrane protein expressed on the apical surface of alveolar epithelial type I cells in rat lungs (10). In various rat models of acute lung injury, the amount of RTI40 recovered in bronchoalveolar lavage fluid was associated with the extent of morphological damage to alveolar epithelial type I cells (26, 27). However, the inability to detect and quantify damage to other epithelial cell types has hampered our understanding of how toxic agents damage the lung.

A number of integral membrane proteins are highly expressed on alveolar epithelial type II and Clara cells, including p172 (14), aminopeptidase N (13), alkaline phosphatase (12), and pneumocin (24). However, these proteins may not be suitable as biochemical markers of type II or Clara cell injury. Specifically, aminopeptidase N and alkaline phosphatase are also expressed on inflammatory cells (12, 15), whereas the potential of p172 and pneumocin to act as cell-specific markers of injury has not yet been evaluated in lung injury models. We have developed a monoclonal antibody (MAb) against the apical surface of rat alveolar epithelial type II and Clara cells, referred to as the MMC4 MAb.

As the first step toward developing a biochemical marker of type II and Clara cell injury, we characterized the MMC4 antigen. First, we determined the tissue distribution of the MMC4 antigen in adult rat tissues. Second, we determined whether the MMC4 antigen was a protein and, if so, whether it was a peripheral or integral membrane protein. Third, because we were unable to obtain a molecular weight by Western blotting, we determined the sedimentation coefficient ($S_{20,w}$) of the MMC4 antigen by glycerol gradient centrifugation. Fourth, we determined whether the MMC4 antigen is regulated during development. Our data demonstrate that the MMC4 antigen is a novel integral membrane protein that is also expressed in the kidney and intestine. Our data suggest that the

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MMC4 antigen may be a useful marker of type II and Clara cell injury in models of lung injury.

METHODS

Alveolar epithelial type II cell isolation. Type II cells were isolated from the lungs of male Sprague-Dawley specific pathogen-free rats (Charles River Laboratories, Irvine, CA) by previously described methods (10). Briefly, the lungs were digested by an intratracheal instillation of elastase (Boehringer Mannheim, Indianapolis, IN) followed by differential adherence of the cells on bacteriological plastic plates coated with rat immunoglobulin G (IgG; Sigma, St. Louis, MO). All animal procedures used in this study were approved by the University of California, San Francisco Animal Care Committee or under a license from the Department of Health (Ireland).

Immunization of mice. Female BALB/c mice (9 wk old) were obtained from Bantin Kingman (San Mateo, CA). Mice were anesthetized with pentobarbital sodium (45 mg/kg) and immunized by intraperitoneal injection of 2 × 10⁶ alveolar epithelial type II cells. Thirteen days later, mice were boosted with type II cells (1 × 10⁶ cells) also injected directly into the spleen. Sera from immunized mice were tested 3 days later (day 15) on thin frozen lung sections for reactivity against alveolar type II cells; type II cells were identified as cuboidal cells containing inclusion bodies located in the corners of alveoli (8, 10). The spleen from the mouse showing the strongest staining against type II cells was used for the production of MAbs.

Production of MAbs. Spleen cells were fused with SP/0 cells (American Type Culture Collection) with polyethylene glycol 4000 following a standard technique (16). Supernatants from hybridomas were tested for reactivity against type II cells by indirect immunofluorescence on sections of frozen rat lung (2 μm thick). Positive hybridomas were recloned three times by serial dilution to ensure a single clone.

The isotype of MMC4 MAb was determined with an ISO-1 mouse MAb isotyping kit (Boehringer Mannheim).

Tissue fixation for thin frozen sections. Lungs from adult rats were fixed by intratracheal instillation of paraformaldehyde (4% wt/vol) prepared in phosphate-buffered saline (PBS). After 2 h of fixation, 2-mm³ pieces of lung were cryoprotected in 15% (wt/vol) sucrose for 18 h at 4°C. Blocks of lung tissue were frozen in liquid nitrogen-cooled Freon 22, and 2- or 5-μm lung sections were cut in a cryostat (Sorvall MT 6000).

Adult kidney and intestines were fixed with the same basic protocol as for lung tissue except for the following minor modifications. The kidneys were removed from the rats, sliced into thin sections, and then immersed in 4% (wt/vol) paraformaldehyde in PBS. For adult and neonatal intestines, the contents were removed and the lumen was filled with 4% (wt/vol) paraformaldehyde. The intestine was then immersed in 4% (wt/vol) paraformaldehyde and treated with 20% (wt/vol) proteinase K to remove any residual glycosidase activity before tissue digestion. The extent of MMC4 MAb binding to proteinase K-digested lung and kidney PNSs was assessed by dot blot analysis. The sensitivity of rat lung RTI40 to proteinase K digestion was run as a positive control.

Proteinase K treatment. Rat lung and kidney PNSs were incubated with 10 mg/ml of proteinase K (Boehringer Mannheim) in 50 mM HEPES, pH 7.4, containing 0.15 M NaCl for 24 h at 56°C. Proteinase K solution was preincubated at 37°C to remove any residual glycosidase activity before tissue digestion. The extent of MMC4 MAb binding to proteinase K-digested lung and kidney PNSs was assessed by dot blot analysis. The sensitivity of rat lung RTI40 to proteinase K digestion was run as a positive control.

MMC4 antigen detergent solubilization studies. To determine whether the MMC4 antigen could be solubilized without loss of MMC4 MAb binding, lung and kidney PNSs were solubilized at 0°C for 30 min in either SDS, C₁₂E₈ (octaethyleneglycol mono-n-dodecyl ether; Calbiochem), CN Bio-Sciences, Nottingham, UK), Triton X-114, or β-octylglucoside (Calbiochem). Lung and kidney PNSs were solubilized at a 1:10-1:100 dilution of PNS in detergent per milligram of protein. The sensitivity of rat lung RTI40 to proteinase K digestion was run as a positive control.

MMC4 antigen may be a useful marker of type II and Clara cell injury in models of lung injury.
to the detergent-insoluble pellet and supernatant fractions was compared with that in nontreated PNSs.

Sodium carbonate wash. Lung and kidney membrane fractions were obtained by centrifugation of PNSs at 541,000 g_{max} for 10 min at 4°C. The membrane pellet was retained and washed three times with 0.1 M sodium carbonate solution, pH 11 (19), or 0.15 M NaCl. The washed membrane pellet was resuspended in 10 mM Tris-HCl, pH 8.2, with 0.15 M NaCl [Tris-buffered saline (TBS)], and the amount of MMC4 antigen recovered in the washed membranes was determined by dot blot analysis.

Triton X-114 phase separation. Triton X-114 was precondensed as described by Bordier (6) and used as a 10% (wt/vol) stock solution. Kidney PNS was solubilized in Triton X-114 (detergent-to-protein ratio of 10:1) for 30 min on ice. The Triton X-114-solubilized PNS was then centrifuged at 541,000 g_{max} for 10 min, and the supernatant was retained. The supernatant was warmed to 30°C for 5 min, and the detergent-rich phase, which forms at the cloud point of Triton X-114, was separated by centrifugation (28). The amount of detergent-insoluble pellet and supernatant fractions was obtained by centrifugation of PNSs at 541,000 g_{max} for 10 min, and the supernatant was retained. The supernatant was warmed to 30°C for 5 min, and the detergent-rich phase, which forms at the cloud point of Triton X-114, was separated by centrifugation (28). The amount of MMC4 antigen recovered in the washed membranes was determined by dot blot analysis.

Preparation of rat kidney plasma membranes. Differential centrifugation through Dextran 6000 was used to obtain a kidney microsomal fraction (3). Adult rat kidney PNS (12 ml) was first centrifuged at 26,500 g_{av} for 20 min to yield a postmitochondrial supernatant. The postmitochondrial supernatant was then centrifuged at 541,000 g_{max} for 10 min to yield a microsomal pellet. The microsomal pellet was resuspended in 10 mM Tris-HCl, pH 8.6, containing 5 mM magnesium sulfate and 0.1 mM phenylmethylsulfonyl fluoride with a glass Dounce homogenizer and dialyzed for 18 h against the Tris-Mg^{2+} buffer. The dialyzed microsomal fraction was layered onto a 20% (wt/vol) dextran step and centrifuged at 541,000 g_{max} for 15 min. The plasma membrane fraction at the Tris-Mg^{2+}-dextran interface was collected and solubilized in TBS containing C_{12}E_{8} (0.01% wt/vol).

Glycerol gradient estimation of MMC4 antigen sedimentation coefficient. Glycerol density gradient sedimentation was performed at 4°C on a 2.5-ml linear gradient of 8–35% (wt/vol) glycerol in TBS containing C_{12}E_{8} (0.01% wt/vol) (1). Kidney plasma membrane [80 μg of protein in 0.05 ml of TBS containing 0.01% (wt/vol) C_{12}E_{8}] was layered onto the glycerol gradient and centrifuged for 12 h at 166,000 g_{av} (Beckman model TLS 55 swinging bucket rotor).

The total amount of protein and the amount of MMC4 antigen were determined in fractions collected from the gradient. To determine the S_{20,w} of the MMC4 antigen, proteins with known S_{20,w} values (i.e., catalase 11.7, γ-globulin 7.3, BSA 4.4, and carbonic anhydrase 3.3) (32) were used to calibrate the gradient.

Developmental expression of the MMC4 antigen. The amount of the MMC4 antigen in lung, kidney, and intestine was measured in fetal (days 16, 19, and 21) and neonatal (days 1, 2, 5, and 8) rats. Timed-pregnant,Sprague-Dawley rats (n = 3/time point) were obtained from University College (Dublin, Ireland) – 1 wk before giving birth. Fetal day 0 was defined as the day a vaginal plug was obtained. Pregnant dams and neonatal rats were anesthetized with pentobarbital sodium (45 mg/kg body wt). Fetuses were obtained by laparotomy. Fetal tissues from one mother were pooled. All tissues were stored at −80°C for analysis at a later point. Data are expressed as relative densitometry units per milligram of protein.

Statistics. Data are expressed as means ± SE. Comparison between samples was analyzed with one-way ANOVA, with Student-Newman-Keuls posttest analysis. P < 0.05 was considered to be significant. Tests were performed with GraphPad InStat version 3.00 for Windows 96.

RESULTS

Immunofluorescence location of the MMC4 antigen in adult lung tissue. The MMC4 MAb (isotype IgG2a) bound the apical surface of epithelial cells in the corners of alveoli (Figs. 1A and 2A). These epithelial cells were judged to be type II cells by the presence of lamellar bodies (10) and from their location between alveolar epithelial type I cells (Figs. 1A and 2A) (8). The MMC4 MAb also bound bronchiolar epithelial cells in the airways (Figs. 1A and 2C). These airway epithelial cells were judged to be Clara cells because they had

![Image](http://ajplung.physiology.org/)
a protruding apical membrane and no cilia (Fig. 2, C and D) (31). The MMC4 MAb did not stain type I cells, macrophages, blood vessels, ciliated cells, or cells beneath the surface bronchiolar epithelial layer (Figs. 1C and 2C).

The MMC4 antigen is present in the kidney and small intestine. Relative to lung, the MMC4 antigen was highly expressed in the kidney (14,079 ± 3,317 vs. 937 ± 271 RDU/mg protein for lung). Immunofluorescence microscopy on thin frozen sections showed that the MMC4 MAb bound the apical surface of selective tubules in the kidney cortex (Fig. 3, A and B). The MMC4-positive tubules were determined to be proximal tubules by the presence of a brush border at the apical surface of tubule epithelial cells (7). The MMC4 MAb did not stain distal or conducting tubules of the cortex or glomeruli (Fig. 3, A and B).

The MMC4 antigen was also highly expressed in the small intestine (1,628 ± 271 RDU/mg protein in the small intestine vs. 937 ± 271 RDU/mg protein in the lung).

Fig. 2. High-magnification view of the immunofluorescence localization of the MMC4 MAb in adult rat lung. A: MMC4 MAb bound to the apical surface of cuboidal epithelial cells (red; arrow) located between RTI40-positive cells (green; type I cells). B: corresponding phase-contrast image. C: MMC4 MAb also bound to the apical surface of nonciliated bronchiolar epithelial cells (red; arrows). D: corresponding phase-contrast image demonstrating ciliated cells (*). These data are consistent with the localization of the MMC4 antigen on the apical surface of both alveolar epithelial type II and Clara cells. Original magnification, ×1,000.

Fig. 3. Immunofluorescence localization of the MMC4 MAb in adult rat kidney and small intestine. Kidney and small intestine sections were stained with the MMC4 MAb (red or green). Nuclei were stained with Hoechst DNA dye (blue). A: MMC4 MAb bound to selected tubules in rat kidney cortex (C; red; *) but did not bind tubules in the medulla region (M). B: higher-magnification view of rat kidney demonstrating that the MMC4 MAb bound to the apical surface of epithelial cells of proximal tubules in the kidney cortex (green; corresponding phase-contrast image not shown); the adjacent glomerulus (G) was negative. C: MMC4 MAb bound to epithelial cells along the tip and middle regions of villi in the small intestine (red; short arrows). Epithelial cells lining the crypts (long arrows) and other regions of the villi were negative. D: higher-magnification view of a villus demonstrating that the MMC4 MAb bound the apical surface of the epithelial cells (green; long arrows). MMC4 MAb fluorescence stopped abruptly at the tip of the villus (short arrows) at the location of a goblet cell (corresponding phase-contrast image not shown). Cells inside the villus [i.e., lamina propria (LP)] were negative. Original magnifications: ×100 in A and C; ×500 in B and D.
the lung). Immunofluorescence analysis showed that the MMC4 MAb stained the apical surface of intestinal villi epithelial cells (Fig. 3, C and D). The MMC4 MAb did not stain goblet cells, inside the lumen of the villi (i.e., lamina propria), or crypt cells (i.e., stem cells, undifferentiated epithelial cells, goblet cells, or Paneth cells; Fig. 3, C and D). Along different regions of the small intestine (i.e., ileum, jejunum, and duodenum), the MMC4 antigen was expressed uniformly (data not shown) and with the same immunofluorescence distribution (i.e., tip and middle epithelial cells of the villi; Fig. 3C).

Although by immunofluorescence analysis, no specific MMC4 staining was detectable in the brain or the eye, by dot blot analysis, the MMC4 antigen was present at very low levels in the brain, eye, and stomach (i.e., 20–45 RDU/mg protein). The MMC4 antigen was not detectable by dot blot analysis in the spleen, liver, heart, testis, trachea, or serum.

The MMC4 antigen is a protein. Heating lung and kidney tissue extracts (PNS) for 18 h at 56°C significantly decreased the extent of MMC4 MAb binding as assessed by dot blot analysis (Table 1). The addition of proteinase K reduced the MAb binding to almost zero (Table 1). The sensitivity of the MMC4 antigen to both heat denaturation and proteolysis suggests that it is a protein.

The MMC4 antigen was not detectable by Western blot analysis. It was not possible to obtain a molecular weight for the MMC4 antigen by SDS-PAGE and Western blotting under nonreducing or reducing conditions. Specifically, we did not detect a signal with lung, kidney, or intestine PNSs, kidney plasma membrane fractions, or dot blot-positive fractions obtained after glycerol gradient sedimentation (data not shown). These data suggest the MMC4 MAb does not recognize the SDS-solubilized antigen.

Detergent solubilization of the MMC4 antigen. Detergent solubilization was required to remove the MMC4 antigen from lung and kidney membrane fractions (541,000 gmax pellet from PNS). Nonionic detergents such as Triton X-114 and C12E8 solubilized the MMC4 antigen without loss of MMC4 MAb binding as assayed by dot blot analysis under nondenaturing conditions. However, exposure to anionic detergents such as SDS interfered with the binding of the MMC4 MAb (i.e., the amount of MMC4 binding in kidney PNS was reduced by 80% at a SDS detergent-to-protein ratio of 5:1).

MMC4 antigen is an integral membrane protein. Most of the MMC4 antigen (85–90%) was detected in the insoluble fraction after high-speed centrifugation (541,000 gmax) of lung and kidney PNSs. Moreover, when lung and kidney membrane fractions were subsequently washed with sodium carbonate to remove peripheral membrane proteins and membranes-adsorbed soluble proteins, the MMC4 antigen specific activity was not reduced. In addition, the MMC4 antigen partitioned in the detergent-rich phase after Triton X-114 phase separation (Table 2). Both the behavior of the MMC4 antigen after a sodium carbonate wash and phase separation into Triton X-114 suggests that the antigen is an integral membrane protein (6, 28).

Sedimentation analysis of the MMC4 antigen. The S20,w of the C12E8-solubilized MMC4 antigen was determined by glycerol gradient sedimentation (1). Plasma membrane fractions from kidney and lung PNSs were isolated over a step gradient of Dextran 6000 (3). The MMC4 specific activity of plasma membrane fractions was typically four- to fivefold greater than the specific activity of PNSs.

Plasma membrane fractions were solubilized with C12E8 and layered onto glycerol gradients to separate the proteins. After centrifugation, the MMC4 antigen was detected by dot blot analysis in fractions 1–4 at the bottom of the gradient (Fig. 4A). When the gradients were calibrated with standard proteins, the MMC4 antigen had an S20,w value of 10.1. To determine whether the S20,w 10.1 form of the MMC4 antigen was associated noncovalently with other proteins, we treated fraction 2 with 4 M urea to disrupt any noncovalent bonds. Urea treatment did not reduce the extent

### Table 1. Effect of temperature and proteinase K treatment on MMC4 MAb binding to lung and kidney

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DU/μg Protein</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>0.29 ± 0.04</td>
<td>100</td>
</tr>
<tr>
<td>56°C</td>
<td>0.05 ± 0.01</td>
<td>16</td>
</tr>
<tr>
<td>56°C + proteinase K</td>
<td>Not detectable</td>
<td>0</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>2.56 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td>56°C</td>
<td>0.005</td>
<td>0.2</td>
</tr>
<tr>
<td>56°C + proteinase K</td>
<td>0.04 ± 0.02</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Values are means ± SD from 1 experiment performed in triplicate. MAb, monoclonal antibody; DU, densitometry units.

### Table 2. Distribution of MMC4 antigen after temperature-induced Triton X-114 phase separation

<table>
<thead>
<tr>
<th></th>
<th>Triton X-114-Solubilized Homogenate</th>
<th>Triton X-114-Solubilized Supernatant</th>
<th>Triton X-114-Insoluble Pellet</th>
<th>Triton-X-114-Rich Phase After Heating Supernatant</th>
<th>Triton X-114-Poor Phase After Heating Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC4, DU/μg protein</td>
<td>6.29</td>
<td>6.33</td>
<td>6.48</td>
<td>16.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Recovery of MMC4 antigen, %</td>
<td>100</td>
<td>86.4</td>
<td>2.24</td>
<td>64.9</td>
<td>27.1</td>
</tr>
</tbody>
</table>

(75% of Triton X-114-solubilized supernatant) (31% of Triton X-114-solubilized supernatant)

Data are from 1 representative experiment.
of the MMC4 MAb binding compared with that in untreated control samples (data not shown). Moreover, when the urea-treated samples were recentrifuged over a glycerol gradient, the MMC4 antigen was recovered in fractions 18 and 19 at the top of the gradient (Fig. 4, B and C). The MMC4 antigen in fractions 18 and 19 had an $S_{20,w}$ value of 1.66. These data suggest that the MMC4 antigen exists as part of a protein complex that is held together by noncovalent bonds.

Developmental expression of the MMC4 antigen. The amount of the MMC4 antigen per milligram of PNS protein increased 12-fold during lung development (Fig. 5A). Specifically, the amount of MMC4 antigen per milligram of protein increased 3.5-fold from fetal day 16 to postgestational day 1 ($P < 0.05$) and 3.3-fold from postgestational day 1 to adult values ($P < 0.05$; Fig. 5A). RTI40, a marker of alveolar epithelial maturation (33, 34), also increased 12-fold between gestational day 16 and adult values ($P < 0.01$; Fig. 5B). In fetal day 21 lung sections, MMC4 MAb bound the apical surface of single cells in tubules that were lined predominately with RTI40-positive epithelial cells (Fig. 6A). In larger tubules, MMC4-positive cells were flanked by both RTI40-positive and -negative cells (Fig. 6B). On fetal day 21, the MMC4 antigen and RTI40 did not to colocalize on the same epithelial cells.

Fig. 4. Native molecular weight determination of the MMC4 antigen by glycerol gradient sedimentation. A: $C_{12}E_8$ (octaethyleneglycol mono-$n$-dodecyl ether)-solubilized MMC4 antigen (from kidney plasma membranes) sedimented predominantly in the denser fractions (i.e., fractions 1–4) after glycerol gradient sedimentation. Arrows, locations of standard proteins: C, catalase; GG, $\gamma$-globulin; BSA, bovine serum albumin; CA, carbonic anhydrase. B: resedimentation of an MMC4-positive glycerol gradient fraction (fraction 2; Fig. 3A). The MMC4 antigen is predominantly located in the denser glycerol gradient fractions (i.e., fractions 1 and 2). C: resedimentation of an MMC4-positive glycerol gradient fraction after urea treatment (fraction 2). In contrast to Fig. 3B, the MMC4 antigen is now located in the least dense fractions of the gradient (i.e., fractions 18 and 19). DU, densitometry units.

Fig. 5. Expression of the MMC4 antigen during development of the lung, kidney, and intestine. A: lung MMC4. B: lung RTI40. C: kidney MMC4. D: intestine MMC4. The amount of RTI40 in lung tissue was measured as a marker of alveolar maturation. RDU, relative densitometry units; 16, 19, and 21, fetal days; 1, 5, and 8, postgestational days. Values are means ± SE; $n = 3$ experiments.
During rat kidney development, there was an incremental accumulation of the MMC4 antigen per milligram of PNS protein (Fig. 5C). The amount of the MMC4 antigen increased 11-fold between fetal days 16 and 19 (P < 0.01), 1.7-fold between fetal day 19 and postgestational day 5, and 11-fold between postgestational day 5 and adult values (P < 0.05). Immunofluorescence localization of the MMC4 antigen on fetal day 21 revealed that the MMC4 antigen stained the apical surface of epithelial cells within selected tubules in the kidney cortex (Fig. 6C). During development of the rat intestine, the amount of MMC4 antigen increased 20-fold between gestational days 16 and 19 (P < 0.01) and by a further 7.5-fold between fetal day 19 and postgestational day 1. However, the amount of the MMC4 antigen decreased fivefold (P < 0.05) between postgestational day 1 and adult values (Fig. 5D). As determined by immunofluorescence localization, the MMC4 antigen was located on the apical surface of intestinal epithelial cells (Fig. 6D). The pattern of MMC4 MAb binding in fetal day 21 intestine was very similar to staining in the adult small intestine (Fig. 3, C and D).

**DISCUSSION**

The ability to detect and quantify the extent of injury to distinct cells of the alveolar wall would greatly improve our understanding of lung damage induced by different toxic agents. McElroy and colleagues (26, 27) have previously demonstrated that an integral membrane protein, RTI40, can be used to measure type I cell damage. Membrane proteins associated with alveolar type II cells either have not yet been evaluated for their use as biochemical markers of epithelial damage or are unsuitable because they are also expressed on inflammatory cells (12, 15). We have developed a MAb (MMC4) against the apical surface of rat alveolar epithelial type II and Clara cells. The overall objective of our study was to characterize the MMC4 antigen to determine whether it might be a potential marker of cell-selective damage.

The MMC4 antigen is an integral membrane protein. The MMC4 antigen was determined to be a membrane protein by both immunofluorescence and biochemical criteria. The MMC4 MAb bound the apical surface of selective cells in the lung, kidney, and small intestine by immunofluorescence detection on frozen tissue sections (Figs. 1–3). The MMC4 antigen was recovered in the insoluble membrane fraction after a high-speed centrifugation of kidney and lung PNSs. In addition, the specific activity of the MMC4 antigen was concentrated in isolated plasma membranes. The MMC4 antigen was determined to be a protein by denaturation during heat treatment and by susceptibility to protease digestion (Table 1). The epitope recognized by the MMC4 antibody was solubilized in the presence of nonionic detergents such as C12E8 and Triton X-114. The integral membrane nature of the MMC4 antigen was shown by its resistance to removal from membrane fractions by washing with sodium carbonate (19) and by its partitioning into a detergent-rich phase after solubilization in Triton X-114 and phase separation (Table 2).

Several other proteins that are located on the apical plasma membrane of alveolar epithelial type II and Clara cells, for example, aminopeptidase N (13), p172 (14), alkaline phosphatase (12) and pneumocin (24), have been identified. However, the MMC4 antigen may be a novel protein based on its tissue distribution and its solubility in Triton X-114. The MMC4 antigen is not detectable in the adult rat liver, but both aminopeptidase N and pneumocin are present in the liver (24, 29).
The MMC4 antigen is expressed in both the kidney and intestine, whereas p172 is not detectable in either of these tissues (14). Alkaline phosphatase, like the MMC4 antigen, is also located in the kidney and small intestine (reviewed in Ref. 15). However, after Triton X-114 solubilization, alkaline phosphatase is recovered in the insoluble phase (18). In contrast, the MMC4 antigen partitions into a detergent-rich phase after Triton X-114 phase separation (Table 2). Insolubility in Triton X-114 is a common feature of glycosylphosphatidylinositol membrane-anchored proteins such as alkaline phosphatase (18). Therefore, our data also suggest that the MMC4 antigen is not a glycosylphosphatidylinositol-anchored membrane protein.

Unfortunately, we were not able to determine a molecular weight for the MMC4 antigen by SDS-PAGE and Western blotting. However, the MMC4 antigen was solubilized in a nonionic detergent as part of a protein complex (S20, w 10.1) when analyzed by glycerol gradient sedimentation (Fig. 4). Treatment of the MMC4 S20, w 10.1 complex with urea disrupted any noncovalent interactions and produced a smaller form of the MMC4 antigen that had an S20, w value of 1.66 (Fig. 4). Hydrolytic enzymes, located in the brush border of kidney and intestinal tissues, are mostly dimeric proteins held together by noncovalent bonds (21). Our sedimentation data suggest the MMC4 antigen is part of a protein complex, and, in common with other brush border integral membrane proteins (21), disulfide bonds are not required to maintain the complex. However, we do not know whether the MMC4 antigen exists as a multimeric or a heteromeric protein complex.

**The MMC4 antigen is developmentally regulated.**

Many proteins associated with the apical surfaces of epithelial cells are developmentally regulated (2, 4, 14, 20, 30, 33–35). Our study demonstrates that the concentration of lung MMC4 antigen (in RDU/mg protein) increases during fetal and postnatal development (Fig. 5A). The MMC4 antigen was first detected in rat lung tissues on fetal day 16, which is before the morphological maturation of both alveolar epithelial type II and Clara cells (17). Other lung cell-selective proteins, such as RTI40 and aminopeptidase N, are also detected early in development (20, 35). In addition, a number of these cell-selective proteins are coexpressed on the same epithelial cell, suggesting that a common progenitor cell gives rise to different epithelial cell types (35). We did not investigate whether RTI40 and the MMC4 antigen were coexpressed in fetal day 16 lungs. However, by fetal day 21, a point at which type II and I cells are distinguishable from each other by electron-microscopic analysis (17), the MMC4 antigen and RTI40 are expressed on different epithelial cells (Fig. 6, A and B).

Most known type II- and Clara cell-associated proteins are developmentally regulated e.g., surfactant proteins A and B (30), aminopeptidase N (20), alkaline phosphatase (11), p172 (14), and pneumocin (20). The rate at which the MMC4 antigen accumulates during lung development is distinct from surfactant protein A, p172, and alkaline phosphatase (11, 14, 30). Both lung surfactant protein A and p172 are not strongly expressed until fetal day 19 when there is a dramatic increase in both proteins before fetal day 22 (birth) (30, 14). The increased expression of surfactant protein A and p172 is associated with the differentiation of alveolar epithelial type II cells (17, 30). Fetal lung alkaline phosphatase activity also increases rapidly before birth but then decreases during the neonatal period to adult values (11). The developmental expression of the MMC4 antigen in lung tissue most closely mirrors the developmental expression of RTI40 and aminopeptidase N (Fig. 5B) (20). The MMC4 antigen, RTI40, and aminopeptidase N are all detectable on or before fetal day 16 (20, 22, 35). In addition, the concentration of each of these proteins in lung tissue increases ~12-fold between fetal day 16 and adult values (Fig. 5, A and B) (20, 35).

During kidney development, the amount of MMC4 antigen increased dramatically at two different stages (Fig. 5C). The first stage was between fetal days 16 and 19, and the second stage was between postnatal day 5 and adult values. The first phase may be associated with a large (27-fold) expansion of the tubular compartment of the developing kidney (5). The second stage may be associated with an increase in the surface area of the brush border epithelial cells of the proximal tubules (23, 33). Like the MMC4 antigen, the specific activities of kidney brush border enzymes such as alkaline phosphatase and aminopeptidase N also increase rapidly after birth (33). However, unlike the MMC4 antigen, neither alkaline phosphatase nor aminopeptidase N are detected before fetal day 18 in the rat kidney (33).

In contrast to lung and kidney development, intestinal MMC4 antigen expression increased during fetal life, then decreased shortly after birth to adult values (Fig. 5D). Similar patterns of expression have been reported for known intestinal brush border enzymes (e.g., alkaline phosphatase and aminopeptidase N) (2, 4). The peak of MMC4 expression on neonatal day 1 (Fig. 5D) is consistent with morphological data showing maturation of rat intestinal villi on fetal day 21 (11). Specifically, the villi are long and cylindrical and the epithelial cells are columnar with microvilli (11). As in lung and kidney development, the MMC4 antigen was detected early in gestation before morphological maturation of the intestinal villus epithelial cells (2, 11).

In summary, we have described a new MAb that recognizes a novel integral membrane protein located on the apical surface of selective epithelial cells in the lung, kidney, and small intestine. We have also demonstrated that the MMC4 antigen is developmentally regulated and that expression of the MMC4 antigen is restricted to specific epithelial cell types on fetal day 21 in lung, kidney, and intestinal tissues. The MMC4 MAb will be a useful tool for analyzing epithelial cell phenotype in lung injury and developmental studies. Furthermore, like RTI40, we expect that the MMC4 antigen might be a useful biochemical marker of cell-selective damage in models of lung injury.
We thank Profs. Christopher Haslett and Christopher Bell for support; Maria Maglio and Hadas Millo for excellent technical assistance; Mark Lawson for help with preparation of the immunofluorescent images; Ian Dransfield, Adriano Rossi, Graham Thomas, and Joseph Gray for comments on the manuscript; and Kirsty Tyrrell for proofreading the manuscript.

This work was funded by the California Lung Association, the Health Research Board (Ireland), and the Medical Research Council.

REFERENCES


